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4 August 1993

SGRD-UIV (70-1r)

MEMORANDUM FOR Chief, Extramural Research Programs

SUBJECT: ILIR Proposal

Request approval of the attached ILIR Research Proposal.

Encl

JEFFREY D. CHULAY
COL, MC
Chief, Virology Division

EXHIBIT G

ILIR PROPOSAL

Development of a Venezuelan Equine Encephalitis Virus Replicon as a Universal Vaccine Vector.

INTRODUCTION AND BACKGROUND

Because of serious deficiencies in the existing vaccines for Venezuelan equine encephalitis virus, USAMRIID has initiated a vaccine development program to produce an improved live vaccine. This program is using recombinant DNA technology which allows the generation of vaccine candidates with precisely defined characteristics. This effort, which is a collaborative project between elements of the Virology Division at USAMRIID and an extramural contractor at the University of North Carolina, has been successful in the generation of several candidates which are currently being tested in various animal models. The basic approach has been to use site-directed mutagenesis techniques to insert multiple, defined, attenuating mutations into full-length cDNA clones of VEE virus from which infectious vaccine candidates can be recovered by transfection of transcribed RNA into susceptible cells.

This ability to manipulate the genome of positive-stranded RNA viruses such as VEE virus and to recover modified, but infectious virions, allows a control over virus replication, transcription, and protein expression to an extent not previously possible. During the vaccine development studies, it also became apparent that VEE virus was much more tolerant of major changes in its genome (deletions, substitutions, etc.) than had been assumed. This ILIR study proposes to take advantage of the new technology and molecular constructs utilized in the VEE vaccine development program, the apparent pliability of the VEE genome, and recent observations made by others to develop a new vaccine vector system with unique properties. Such a system is expected to be useful in the development of a variety of viral, bacterial, protozoal, and toxin vaccines within both the BDRP and the IDRP.

Recently, Liljestrom and Garrof (1) have described a unique expression system using the related alphavirus, Semliki Forest virus (SFV), which is capable of very high expression levels of heterologous genes in vitro (up to 20% of the total cell protein). In this system, the gene to be expressed is inserted in place of the SFV structural genes in a transcription plasmid containing an otherwise full-length copy of the SFV genome. RNA transcribed from this plasmid in vitro contains the viral replication and transcription functions intact, and when transfected into susceptible cells is replicated, transcribed, and translated.

This replicating RNA, or "replicon", can be packaged into normal virus parti
if the SFV structural protein genes are provided on a second ("helper") replicon

which is cotransfected with the first. The helper replicon need not have the nonstructural genes for its own replication and transcription as those functions are provided, in trans, by the first replicon. Furthermore, as the RNA packaging signal for alphaviruses is contained within a nonstructural gene, only the replicon RNA containing the nonstructural genes and the foreign gene is encapsidated and packaged into viral particles. The viral coat proteins provide the replicon with the same cell specificity and infectivity as the normal virus. However, as the structural protein genes are absent, the infectious particles released from the replicon-infected cells are capable only of a single round of abortive infection in subsequently infected cells during which the foreign gene is efficiently expressed. This inability for sustained replication, as well as the high level expression achieved, should make an alphavirus replicon an exceptionally safe and versatile vaccine vector system.

The potential use of the SFV replicon as a vaccine vector was not addressed by Liljestrom and Garrof, and would be problematic as 1) homologous recombination has been observed between their recombinant and helper replicons resulting in the generation of wild type SFV which is a human pathogen, and 2) the SFV replicon was not designed or produced as a human use product. The studies proposed here will construct a replicon system similar to the SFV system, but will use one of the genetically defined, multiply attenuated, VEE vaccine candidates developed in the ongoing program summarized above. This virus has already been shown to be attenuated in mice, hamsters, and horses (2,3) and is being developed as a human use product. In addition, helper replicons will be constructed in such a way as to preclude homologous recombination with the expression replicon and consequent generation of virulent virus.

Specific Research Objectives:

1. Construction of the VEE virus replicon and helper plasmids

These plasmids will be constructed from the previously described full-length cDNA clone of VEE virus in the pV3507 or other attenuated derivative plasmid (2,4) by introduction of site-specific mutations. The helper plasmid will retain the 5'- and 3'-terminal nucleotide sequences of the wild type viral genome (which contain the necessary signals for replication and transcription), but will contain only the genes for the viral structural proteins. The replicon plasmid will contain the 5' and 3' terminal sequences and the nonstructural genes, but the structural protein genes will be deleted and the plasmid will instead contain only the promoter sequences necessary for transcription of the 26S subgenomic mRNA followed by a multiple cloning site into which a variety of genes for relevant antigens can be subcloned.

In order to minimize the possibility of recombination, we will develop also a series of helper replicon constructions or other "packaging systems" which will preclude the generation of infectious virus by recombination. A number of possibilities will be examined including, 1) helper replicon systems in which the structural genes are contained in multiple molecules, or under control of the nonstructural initiation codon, 2) packaging the replicon in SF9 cells coinfected with a baculovirus construct expressing the VEE structural proteins, and 3) packaging in mammalian cells expressing the structural proteins from inducible expression systems.

2. Construction of recombinant replicons and in vitro expression

Replicons will be generated with genes encoding either a reporter gene, chloramphenicol acetyl transferase (CAT), or genes encoding immunogens from viruses known to require B cell (bunyaviruses or alphaviruses) or T cell (arenaviruses) effector mechanisms for protection. In addition, genes encoding two malaria sporozoite surface proteins (P. yoelii) will be studied in collaboration with investigators at the Naval Medical Research Unit, and the botulinum toxin C fragment will be studied in collaboration with Dr. John Middlebrook of the Toxinology division.

The construct with the CAT gene will be used to characterize the in vitro expression potential of the replicon system in BHK cells following direct transfection of the RNA. Packaging of replicon RNA and release of particles containing the replicon RNA will be monitored by gradient centrifugation and antigen capture elisa assays with existing monoclonal antibodies. The number of "infectious units" (IU), i.e. particles containing the replicon RNA and capable of binding and penetrating susceptible cells, will be determined by quantitating immunofluorescent foci, and all preparations will be checked for the appearance of infectious virus by standard plaque assays.

3. Replicon expression, immunogenicity, and protective capacity in vivo

Following optimization of in vitro expression and packaging, replicon particles expressing genes of interest will be inoculated into appropriate rodent hosts at doses of 10^2 to 10^9 IU as a test of the vaccine vector potential of these constructs. Animals will be inoculated subcutaneously, intramuscularly, and intranasaly and monitored for seroconversion by elisa and neutralization tests. Animals will then be challenged with virulent organisms to determine the level of protection achieved. As even 10^9 viral particles contain only 3 ng of viral protein, it is unlikely that the animals would respond immunologically to the VEE structural proteins contained in the input particles alone. However, this assumption will be tested directly by 1) monitoring the immune response to the VEE structural proteins, and 2) by sequentially inoculating animals with replicon particles containing genes for unrelated antigens and comparing the immune responses to uninoculated animals.

Significance

These studies are designed to develop a safe vaccine vector system capable of high level expression to deliver heterologous protective immunogens of viral, parasitic or bacterial origin. Assuming that immunization with the vectored gene product is achieved with packaged replicon doses of 10^9 IU or less, the same constructs could be used repeatedly in the same individual to deliver booster immunizations or unrelated immunizations as seroconversion to the replicon packaging proteins should be insignificant. A unique aspect of this vector system is the restriction to a single round of cell infection which will impart a significant safety margin and allow a degree of control on the immunization process not possible with standard live-attenuated or vector-based vaccines. Similarly, as infectious virus will not be generated in vaccinees, transmission of virus to unvaccinated individuals or to invertebrate vectors will be prevented.

References

1. Liljestrom, P. and H. Garoff. A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. Biotechnology 9:1356-1361.

2. Davis, N.L., Powell, N., Greenwald, G.F., Willis, L.V., Johnson, B.J.B., Smith, J.F. and Johnston, R.E. (1991). Attenuating mutations in the E2 glycoprotein gene of Venezuelan equine encephalitis virus: Construction of single and multiple mutants in a full-length cDNA clone. Virology, 183:20-31.

3. Johnston, R. E., and J. F. Smith. 1986. Selection for Accelerated/Penetration in Cell Culture Co-Selects for Attenuated Mutants of Venezuelan Equine Encephalitis Virus. Virology 162:437-443.

4, Davis, N. L., L. V. Willis, J. F. Smith, and R. E. Johnston. 1989. In Vitro Synthesis of Infectious Venezuelan Equine Encephalitis Virus RNA from a cDNA Clone: Analysis of a Viable Deletion Mutant. Virology 171:189-204.

Budget (FY93)

Supplies	$2200.00	
Equipment	none	
Personnel	(August/September)	
Parker, M.	10%	$1248.00
Glass, P.	10%	$482.